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*Published in:*

Journal of Trace Elements in Medicine and Biology

*Link to article, DOI:*

[10.1016/j.jtemb.2018.02.005](https://doi.org/10.1016/j.jtemb.2018.02.005)

*Publication date:*

2018

*Document Version*

Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*

Sele, V., Ørnsrud, R., Sloth, J. J., Berntssen, M. H. G., & Amlund, H. (2018). Selenium and selenium species in feeds and muscle tissue of Atlantic salmon. *Journal of Trace Elements in Medicine and Biology*, 47, 124-133. <https://doi.org/10.1016/j.jtemb.2018.02.005>

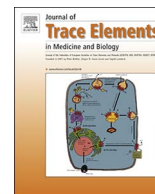
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## Selenium and selenium species in feeds and muscle tissue of Atlantic salmon

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## ARTICLE INFO

## Keywords:

Selenium  
Supplementation  
Speciation analysis  
Feed legislation  
Fish

## ABSTRACT

Selenium (Se) is an essential element for animals, including fish. Due to changes in feed composition for Atlantic salmon (*Salmo salar*), it may be necessary to supplement feeds with Se. In the present work, the transfer of Se and Se species from feed to muscle of Atlantic salmon fed Se supplemented diets was studied. Salmon were fed basal fish feed (0.35 mg Se/kg and 0.89 mg Se/kg feed), or feed supplemented either with selenised yeast or sodium selenite, at low (1–2 mg Se/kg feed) and high (15 mg Se/kg feed) levels, for 12 weeks. For the extraction of Se species from fish muscle, enzymatic cleavage with protease type XIV was applied. The extraction methods for Se species from fish feed were optimised, and two separate extraction procedures were applied, 1) enzymatic cleavage for organic Se supplemented feeds and 2) weak alkaline solvent for inorganic Se supplemented feeds, respectively. For selenium speciation analysis in feed and muscle tissue anion-exchange HPLC-ICP-MS for analysis of inorganic Se species and cation-exchange HPLC-ICP-MS for analysis of organic Se species, were applied. In addition, reversed phase HPLC-ICP-MS was applied for analysis of selenocysteine (SeCys) in selected muscle samples. The results demonstrated that supplemented Se (organic and inorganic) accumulated in muscle of Atlantic salmon, and a higher retention of Se was seen in the muscle of salmon fed organic Se diets. Selenomethionine (SeMet) was the major Se species in salmon fed basal diets and diets supplemented with organic Se, accounting for 91–118% of the total Se. In contrast, for muscle of salmon fed high inorganic Se diet, SeMet accounted for 30% of the total Se peaks detected. Several unidentified Se peaks were detected, in the fish fed high inorganic diet, and analysis showed indicated SeCys is a minor Se species present in this fish muscle tissue.

## 1. Introduction

Selenium (Se) occurs naturally in the terrestrial and marine environment. Marine fish, e.g. Atlantic cod (*Gadus morhua*), Greenland halibut (*Reinhardtius hippoglossoides*) and Atlantic herring (*Clupea harengus*) generally contain high levels (0.25–1.4 mg/kg ww) of total Se [1]. Selenium levels found in fish feed can be ascribed to high levels of Se in marine feed ingredients, mainly fish meal. Today's feed (in Norway) for Atlantic salmon (*Salmo salar*) contains an average of 0.6 mg Se/kg feed (ranging from 0.3 to 1.1 mg Se/kg, n = 50) [2], and during the past 15 years a trend towards lower levels of Se in Atlantic salmon feed has been reported [3] and ascribed to the increased replacement of fish meal with plant ingredients. Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) fed plant based diets have been shown to contain lower muscle or whole body Se concentrations compared to fish fed marine based diets [4–6]. Reduced levels of Se in fish due to lower levels of Se in feed emphasise that it may be necessary to supplement Se to the feeds to meet the physiological requirement of the

farmed fish.

In Europe, the use of feed additives is regulated through the European feed legislation. The current maximum limit for total Se in animal feeds including fish feed has been set at 0.5 mg/kg feed (Council Directive 70/524/EC and amendments). Recently, the European Food Safety Authority (EFSA) has issued several scientific opinions on the use of organic selenium-yeast forms as feed additives and based on the apparent higher bioavailability of organic Se compared to inorganic Se concluded that the supplementation level should be limited to a maximum of 0.2 mg/kg feed to ensure consumer safety [7–10]. Subsequently, the European Union recently regulated the use of several Se feed additives, mainly selenised yeasts, at a supplementation level of maximum 0.2 mg Se/kg feed [11–15]. The legislative differentiation between total Se and supplemented organic Se forms warrants the establishment of suitable Se speciation techniques. Furthermore, such methods will help generating data on the occurrence of Se species in feed and fish, which is important for future risk assessments of Se feed additives.

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Selenium species have previously been determined in fish and mussels using ion-pair reversed-phase high pressure liquid chromatography (HPLC) or anion-exchange HPLC coupled to inductively coupled plasma mass spectrometry (ICP-MS) after enzymatic digestion [1,16,17]. Fish feed is a complex matrix consisting of different sources of lipids and proteins and feed additives, which may cause challenges in the analysis of Se species, e.g. during extraction, chromatographic separation and species identification. In a recent study, Godin et al. [18] applied an extensive approach for the determination of Se species in feed and in whole body of rainbow trout fry. Inorganic Se species were determined in feed using anion-exchange HPLC-ICP-MS following extraction with sodium hydroxide, while selenomethionine (SeMet) was determined in feed and fish using anion-exchange HPLC-ICP-MS following enzymatic extraction [18]. For the determination of selenocysteine (SeCys), a derivatisation step was applied prior to enzymatic extraction to stabilise this Se species for analysis using reversed-phase HPLC-ICP-MS [18].

In fish, supplemented organic Se forms appear to have a higher bioavailability and tissue accumulation compared to supplemented inorganic Se forms [6,19]. The upper limit (0.2 mg/kg feed) for supplementation with selenised yeast (organic Se forms) is based on risk assessments of consumer safety [7–10]. In such assessments, documentation of the feed-to-food transfer in long term (> 3 months) feeding trials is essential. However, the assessments were mainly based on experimental data from trials with terrestrial farmed animals and to a lesser degree for farmed fish. Only a few studies on the speciation on farmed marine fish when subjected to different dietary sources of Se in the feeds exist. Godin et al. found through speciation analysis that trout fry fed diets supplemented with selenised yeast contained higher SeMet concentrations compared to fry given diets supplemented with selenite [18]. In contrast, the SeCys concentration in the fry was not affected by the supplementation source of the diets. Furthermore, speciation analysis of whole fry showed that 24–38% of the total Se was not identified [18]. In order to get a better scientific basis for the risk assessment of the use of different Se feed supplements in fish feed, studies need to address the species-related transfer and accumulation in the chain from fish feed to the final aquaculture product.

In the present work, the aim was to study inorganic and organic Se species in fish feed and fish muscle of farmed Atlantic salmon fed diets supplemented with inorganic Se or organic Se at two different inclusion levels and hence, contribute to a better understanding of the species-related transfer of Se in the aquaculture feed chain. Methods for the determination of Se species in feeds and in muscle of Atlantic salmon were established in order to reach this goal, with a focus on optimizing the extraction of Se species.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals used were analytical grade quality or better. Milli-Q water (18.2 MΩ cm, EMD Millipore Corporation, MA, USA) was used for sample preparation and analysis. Seleno-DL-methionine (SeMet, ≥99% purity), sodium selenate (Se(VI), ≥98% purity), sodium selenite (Se

(IV), 99% purity), seleno-DL-cystine (SeCys2, ≥98%), protease type XIV from *Streptomyces griseus*, lipase from *Candida rugosa*, ammonium phosphate dibasic, ammonium acetate, tris(hydroxymethyl)amino-methane, urea, dithiothreitol, iodoacetamide, heptafluorobutyric acid (HFBA), phosphoric acid and sodium hydroxide were all obtained from Sigma Aldrich (Oslo, Norway). Methanol (HPLC grade), pyridine, hydrochloric acid, ammonia solution, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, Emsure ACS, ISO, 32% w/w) were obtained from Merck (Darmstadt, Germany). Se-methyl-seleno-L-cysteine (SeMetSeCys ≥98%) and nitric acid (HNO<sub>3</sub>, Trace select, ≥69.0% w/w) were obtained from Fluka (Oslo, Norway). A multi element standard, mercury (Hg), gold (Au), germanium (Ge), rhodium (Rh) and thulium (Tm) standards were obtained from Spectrascan (Teknolab, Ski, Norway). A tuning solution of lithium (Li), yttrium (Y), cesium (Ce) and thallium (Tl) (ICP-MS stock solution, tuning solution B) was obtained from Thermo Fisher Scientific Inc (Waltham, MA, USA). Selenium enriched yeast certified reference material (Selm-1) and TORT-3 (Lobster hepatopancreas) were both obtained from the National Research Council Canada, NRC, Ontario, Canada. Oyster Tissue (SRM 1566 b) was obtained from National Institute of Standards and Technology, Gaithersburg, MD, USA and fish muscle tissue (ERM-BB422) from Institute for Reference Materials and Measurements, IRMM, Geel, Belgium.

### 2.2. Samples

All samples were obtained from a feeding trial where Atlantic salmon were fed diets supplemented with Se. The overall aim of the feeding trial was to establish biomarkers of early toxicity [20], while this work describes the speciation and the species-related transfer of Se in feed and muscle of the fish. In short, inorganic Se (selenite; DMS, Heerlen, The Netherlands) or organic Se (selenised yeast containing SeMet; AlkoSel<sup>®</sup>, Lallemand, Malvern Link, England) were added to a basal diet at two nominal levels; 1.4 and 13 mg/kg feed, respectively, resulting in feeds with either low or high Se concentration (Table 1). The basal low diet had a low inclusion of fish meal (8%) and krill meal (2%), and a low Se level. In addition, the experimental design included a basal diet with a higher inclusion of fish meal (15%) and krill meal (7%). Plant-based protein sources were included in both diets, and the protein level of the diets were balance to similar levels. The measured Se concentrations in the diets are shown in Table 1. The diets were produced by BioMar AS (Trondheim, Norway). Subsamples were shipped to NIFES, homogenised and stored in darkness at 4 °C until further analysis. The feeding trial was conducted at the facilities of EWOS Innovation, Dirdal, Norway. Atlantic salmon were fed the six diets in triplicate tanks (30 fish per tank) for 12 weeks. Muscle samples were taken using a standardised cut; two cm wide cut behind the dorsal fin on the left lateral side of the fish. Skin and bones were removed from the muscle. The samples were stored on ice during sampling and transportation to the laboratory. The samples were then stored at –20 °C. The samples were later thawed, homogenised, freeze-dried, and ground and homogenised before being stored at room temperature prior to analysis.

**Table 1**

Selenium concentrations in diets (mg/kg dw, mean ± SD, n = 3), in muscle tissue (mg/kg ww, mean ± SD, n = 9), and the calculated retention (% , n = 3) of Se in the muscle tissue of Atlantic salmon fed the respective diets. Data points with no letter in common are significantly different (p < 0.05).

Description of diets	Nominal Se concentration (mg/kg dw)	Se concentration in diets (mg/kg dw)	Se concentration in muscle tissue (mg/kg ww)	Retention (%)
Basal low	0	0.35 ± 0.02	0.12 ± 0.01 <sup>a</sup>	15 ± 1 <sup>a</sup>
Inorganic Se low	1.4	1.10 ± 0.03	0.14 ± 0.01 <sup>a</sup>	14 ± 3 <sup>a</sup>
Inorganic Se high	13	15.0 ± 0.5	0.46 ± 0.07 <sup>b</sup>	3.7 ± 0.2
Organic Se low	1.4	2.10 ± 0.05	0.43 ± 0.04 <sup>b</sup>	21 ± 1 <sup>b</sup>
Organic Se high	13	15.0 ± 0.2	3.4 ± 0.2	24 ± 1 <sup>b</sup>
Basal high	0	0.89 ± 0.03	0.22 ± 0.03 <sup>a</sup>	70 ± 4

### 2.3. Determination of total selenium

Diets and freeze-dried muscle tissue were digested using the microwave-acid decomposition method described by Julshamn et al. [21]. Samples were accurately weighed (0.2 g) in (PTFE) vessels and concentrated  $\text{HNO}_3$  (2 mL) and  $\text{H}_2\text{O}_2$  (0.5 mL) were added. The samples were digested in a microwave system (Ethos Pro Milestone, Sorisole, Italy) using the following temperature programme: 1 min at 250 W, 1 min at 0 W, 5 min at 250 W, 5 min at 400 W and 5 min at 650 W. The digests were diluted to a final volume of 25 mL with Milli-Q water.

For determination of total Se concentration in extracts of fish muscle, diets and reference materials the extracts were diluted 25 or 50 times, depending on Se concentration of the sample, with Milli-Q water and 5%  $\text{HNO}_3$ , centrifuged at 2.5g for 5 min, and filtered with 0.45  $\mu\text{m}$ , 25 mm membrane filters with syringe (Merck Millipore).

The sample digests and sample extracts were analysed with an iCAP-Q ICP-MS using collision cell and FAST SC-4Q DX autosampler (both Thermo Fisher Scientific Inc., Waltham, MA USA). An external calibration curve was prepared from freshly prepared multi element solutions, which included Se, diluted to appropriate concentrations by 5% (v/v)  $\text{HNO}_3$  and used for the quantification of Se. The instrument was set in He KED (kinetic energy discrimination) mode for interference removal. A solution of internal standard comprising of Ge, Rh and Tm was added on-line for correction of instrumental drift during the analysis. The instrument was tuned daily using a tuning solution (1 ppb tuning solution, Thermo Fisher, in 2%  $\text{HNO}_3$  and 0.5% HCl) prior to analysis. Plasma power was set to 1550 W, nebulizer gas flow to 1.05 L/min, the auxiliary gas flow to 0.8 L/min, and the He gas (CCT1) flow was 4.6 mL/min. Nickel interface cones were used, and a microflow PFA-ST nebulizer (Thermo Fisher Scientific Inc.) was used. Isotope  $^{78}\text{Se}$  was monitored, and the integration time was 0.1 s.

The results obtained for total Se (average  $\pm$  SD)  $10.6 \pm 0.6$  mg/kg;  $n = 8$  (TORT-3) and  $2.1 \pm 0.1$  mg/kg;  $n = 8$  (Oyster Tissue) agreed well with the certified reference values of  $10.9 \pm 1.0$  mg/kg (TORT-3) and  $2.06 \pm 0.15$  mg/kg (Oyster Tissue), respectively. Selected sample extracts were spiked with known amounts of Se using the standard mixture (Agilent Technology), and the mean recovery ( $\pm$  RSD) of the spiked Se was  $103 \pm 8\%$ ;  $n = 12$ .

### 2.4. Determination of selenium species

#### 2.4.1. Sample preparation

Selenium species were extracted by enzymatic hydrolysis using a non-specific protease (Protease type XIV), by modifying the extraction procedure described elsewhere [1]. Diets and freeze-dried muscle tissues were accurately weighed (0.2 g) in polypropylene tubes and suspended in 2.5 mL of 1 mM ammonium phosphate buffer, pH 7, containing 20 mg protease, and mixed vigorously for 1–2 min. The solutions were left in a water bath (OLS200 Grant Instruments, Cambridge, UK), in the dark and at 37 °C, shaking at 100 rpm/min for 24 h. The samples were then cooled to room temperature, before centrifugation (2.5g for 10 min) followed by filtration using 0.45  $\mu\text{m}$ , 25 mm syringe filters (Merck Millipore). Centrifugal filters (Amicon Ultra-0.5 mL 10 kDa, Merck Life Science AS, Oslo, Norway) were used to separate by centrifugation (14,000g for 20 min) the high molecular weight compounds from the low molecular weight Se species (e.g. SeMet with molecular weight of 196.106 g/mol), as an extra clean-up procedure prior to the chromatographic separation. Based on total Se analysis of extracts analysed pre and post filtration (both 45  $\mu\text{m}$  and Amicon filters), the recovery of Se in filtrated extract was  $102 \pm 16\%$  ( $n = 6$ , data not shown). Also, non-filtrated and filtrated samples were compared by chromatographic analysis, and no differences were observed in the signals or in the chromatographic profiles (data not shown). The phosphate buffer was treated in the same way as the samples and used as a procedural blank sample.

The extraction yield for Se in the diets was further studied using

different extraction solvents, both waters-soluble solvents and a combination of enzymatic digests, on the basal high diet. The diet was accurately weighed (0.2 g) in polypropylene tubes and suspended in 2.5 mL of 1 mM ammonium phosphate buffer, pH 7, and in 2.5 mL 10% methanol in Milli-Q water to extract the water-soluble and polar Se species. For enzymatic digestion, the weighted sample (0.2 g) was extracted by 10 mg, 20 mg and 30 mg protease type XIV in 2.5 mL of the phosphate buffer. For studying the effect of lipase, the diet was first extracted by 20 mg protease (24 h in water bath), following addition of 10 mg lipase (in 2.5 mL phosphate buffer) to the same sample. The sample was left on water bath for additional 24 h. Total Se was determined in the final extract by ICP-MS.

Diets were additionally subjected to a sequential proteolytic extraction procedure, where samples were first extracted by protease following the description above, then after centrifugation (2.5g, 10 min) the soluble extract of the samples were transferred to new tubes using Pasteur pipettes. A fresh batch of protease (20 mg) in phosphate buffer was then added to the insoluble sample residue, shaken vigorously, and left for additional 24 h in water bath. This procedure was repeated twice, and the soluble extracts were determined for total Se by ICP-MS.

The inorganic Se supplemented diets were also extracted using 0.1 M NaOH in MilliQ water for the specific extraction of the inorganic Se species. The diets were accurately weighed (0.2 g) in polypropylene tubes and suspended in 5 mL 0.1 M NaOH and mixed on a rotator (model LD79, Labinto, Breda, The Netherlands) for 12 h. The samples were then centrifuged (2.5g for 10 min), diluted with MilliQ-water, and filtrated using 0.45  $\mu\text{m}$ , 25 mm membrane filters with syringe (Merck Millipore).

Selected muscle samples were extracted, derivatised and analysed for SeCys using the protocol described by Bierla et al. [16], including the procedure for sample defatting, protein denaturation and derivatised by carbamidomethylation. Freeze-dried muscle (0.1 g) was extracted with 2 mL of 7 M urea solution in 0.1 M Tris-HCl buffer, pH 7.5, and sonicated for 20 min. The solution was then added 30  $\mu\text{L}$  0.2 M dithiothreitol (DDT) and 40  $\mu\text{L}$  0.5 M iodoacetamide (both in 0.1 M Tris-HCl buffer, pH 7.5) and incubated for 2 h at 25 °C in the dark. Fresh DDT (375  $\mu\text{L}$ ) was added to the mixture, and the samples were shaken for 1 h. The samples were then diluted with 12 mL of 0.1 M Tris-HCl buffer, and a 500  $\mu\text{L}$  aliquot of protease/lipase solution containing 20 mg protease type XIV was added. The samples were incubated in water bath for 20 h at 37 °C. The samples were centrifuged (2.5g, 10 min), filtered by 0.45  $\mu\text{m}$ , 25 mm syringe filters (Merck Millipore) and freeze-dried.

Standards of CAM-SeCys and CAM-SeMet were prepared by a method described by Jagtap et al. [22], adapted from Dernovics and Lobinski [23]. Standards with concentration of 100 mg Se/L in 4 mL of Tris-HCl (0.05 M, pH 8.6) was heated to 37 °C and purged with Ar(g) for 1–2 min, before 100 mg DDT and 224 mg iodoacetamide (both in 4 mL of 0.05 M Tris-HCl, pH 8.6) were added. The standards were incubated in the dark at 37 °C for 1 h. The reaction was stopped by addition of 180 mg of DDT dissolved in 4 mL the Tris-HCl buffer, and diluted with Milli-Q water to a final volume of 20 mL. The standards were further diluted with water prior to analysis with reversed-phase HPLC-ICP-MS.

#### 2.4.2. Analysis of selenium species using ion-exchange chromatography

Selenium species were analysed by HPLC (1260 HPLC system) coupled with an ICP-MS 7500cx (both Agilent Technologies, Wilmington, Delaware, USA). An octopole reaction cell was applied to the ICP-MS for Se speciation analysis, and the cell conditions were optimised for the reduction of argon-based polyatomic interferences using  $\text{H}_2$  as the reaction gas. The  $\text{H}_2$  flow rate was set to 4 mL/min. A Babington nebulizer was used to insert the sample, and the ICP-MS was tuned prior to analysis using a tuning solution, comprising lithium, yttrium, cesium and thallium. Nickel interface cones were used. Isotopes  $^{78}\text{Se}$ ,  $^{80}\text{Se}$  and  $^{82}\text{Se}$  were monitored, and the integration time was 0.1 s. The HPLC was equipped with a quaternary HPLC pump, an

autosampler, a vacuum degasser system and a temperature column compartment. The HPLC column outlet and the sample insertion system of the ICP-MS were coupled with 0.125 mm (i.d.) PEEK tubing. The instrumental settings are given in Supplementary data, S-Table 1.

Two ion-exchange chromatographic methods based on previous reports [17,24–26] were applied for the Se speciation analyses. The chromatographic conditions were optimised by adjusting the buffer, pH, HPLC column and the elution program. Since the chromatographic resolution of Se species in extracts from food samples is typically difficult to achieve [27,28], it was not possible to obtain baseline separation of all Se species present in a sample using a single chromatographic principle. Therefore, quantitative determination of Se species was achieved by combining anion-exchange and cation-exchange HPLC-ICP-MS analysis.

The Se species identification in the samples was based on the comparison with the retention times of Se species standards. For Se speciation analysis, a strong cation-exchange column (Ionospher 5C, 150 × 3 mm, 5 µm; Agilent Technologies) was used for the analysis of organic Se species, and a strong anion-exchange column (Hamilton PRP-X100, 150 × 4.6 mm, 5 µm, VWR, Radnor, PA, USA) was used for the analysis of inorganic Se species. The mobile phases and chromatographic conditions are given in S-Table 1. As differences were observed in the separation efficiency between muscle and diets when analysed by cation-exchange HPLC-ICP-MS, two different gradient elution programs were applied (S-Table 1). The chromatographic peaks were quantified by external calibration. The retention times for Se species were affected by the sample matrix when analysed by cation-exchange HPLC-ICP-MS. For qualitative assignment of the molecular identity of the Se peaks, sample extracts were spiked with Se standards. The limits of detection (LOD) were calculated as three times the baseline noise of a blank sample, and were 0.007 mg/kg for Se(IV) and 0.005 mg/kg for Se(VI) by anion-exchange HPLC-ICP-MS, and 0.01 mg/kg for SeMetSeCys, 0.03 mg/kg for SeMet and 0.02 mg/kg for SeCys2 by cation-exchange HPLC-ICP-MS.

Certified reference materials for speciation analysis of Se in marine samples are not currently available. The reproducibility of analysis was assessed by analysing the reference materials (Selm-1 and ERM-BB442) in all analytical runs. SELM-1 is certified for SeMet and was therefore used for evaluating the accuracy of SeMet determinations.

#### 2.4.3. Analysis of SeCys by SEC-ICP-MS and reversed-phase HPLC-ICP-MS

Chromatographic analysis for SeCys was conducted using the protocol described by Bierla et al. [16]. Derivatised samples were injected onto a Size exclusion Chromatographic (SEC) column (TSKgel G3000 SWXL, 7.8 × 300 mm, 5 µm) with guard column (6 × 40 mm, 7 µm, both Tosoh Bioscience, Griesheim, Germany). The SEC column was calibrated for the low molecular weight compounds by SeMet standard, and by analysis of sample extracts prior to the fractionation. A Fraction Collector (Analytical Scale, G1364C to 1260 HPLC, Agilent Technologies) were applied for collection the selenoamino acid fractions from replicate injections (n = 3) of same sample was then pooled, freeze-dried, and suspended in Milli-Q water (270 µL).

The selenoamino fractions were further analysed by ion-pair reagent chromatography using 0.1% HFBA as the ion-pair reagent on a reversed-phase column XBridge C18 (4.6 × 250 mm, 5 µm) with guard column (4.6 × 20 mm, 5 µm, Waters, Dublin, Ireland). The presence of SeCys in the sample extracts were determined based on overlap in retention times with CAM-SeCys standard, verified by spike experiments. The mobile phases and chromatographic conditions for the SEC separation and the reversed-phase chromatography are given in S-Table 1.

#### 2.5. Data analyses and statistics

The extraction yield (%) was calculated using the following equation;

$$\text{Extraction yield}(\%) = \frac{\text{Total Se (ng) in soluble extract}}{\text{Total Se (ng) in sample}} \times 100$$

Retention of Se in muscle of Atlantic salmon (%) was calculated using the following general equation for retention (%);

$$\text{Retention}(\%) = \left[ \frac{(C_{\text{muscle, end}} \times \text{biomass muscle, end}) - (C_{\text{muscle, start}} \times \text{biomass muscle, start})}{(C_{\text{diet}} \times \text{Feed intake})} \right] \times 100$$

where  $C_{\text{muscle}}$  and  $C_{\text{diet}}$  are the Se concentration in muscle and diet (mg/kg), respectively. The muscle concentration at start was set to 0 mg/kg. The feed intake is the total amount of feed intake for each tank and was adjusted for excess feed. The muscle biomass was estimated based on the weight of whole fish, assuming that 66% of the weight of the whole fish is muscle [29].

The presented data were evaluated statistically using Dell Statistica® (Dell Inc. (2016). Dell Statistica, version 13. software.dell.com). All datasets were tested for normality and homogeneity using Levene's test. Data sets for Se concentrations in muscle were analysed by nested analysis of variance (ANOVA) followed by a Tukey's honest significant difference (HSD) test to identify any significant differences at different time points. To detect significant differences among treatment groups, data set of retention was analysed using one-way ANOVA with post hoc Tukey's HSD test with a level of  $p < 0.05$  considered significant. All data are given as mean ± standard deviation.

### 3. Results and discussion

#### 3.1. Extraction of selenium in feeds and muscle

When using enzymatic cleavage with protease for the extraction of Se, relative high extraction yields were obtained for Se in the muscle samples, ranging from 90 to 110% (Table 2). The extraction yield for Se in the diets were, however, lower and showed a large variation (37–114%) compared to the muscle samples. As the extraction efficiency of Se is critically dependent on i.e. the sample matrix, type of extraction solvent and the ratio of enzyme to sample [26,30,31], different combinations and levels of protease was applied to the basal high diet to potentially increase the extraction efficiency for Se in the diets. From the use of phosphate buffer for the extraction, it was seen that only minor amounts (5%) of Se was extractable (data not shown). Also, when using aqueous methanol, a polar solvent, the extraction efficiency for Se did not increase, indicating that only minor amounts of Se is water-soluble. These results are consistent with previously studies on Se speciation in fish tissue, where only a minor amount of Se (3–5%) was reported as being water-soluble [32]. Proteolytic enzymes are extensively used for the extraction of selenoamino acids from tissues, e.g. meat [16] and fish muscle [1,22] and, therefore, proteolytic enzymes were applied for the extraction of Se. Different levels of protease were added to the diet (10 mg, 20 mg and 30 mg) to study the effect of amount of enzymes. From the total Se measurements of the digests it was seen that 10 mg protease gave an extraction yield of  $46 \pm 4\%$  (n = 3), 20 mg protease gave  $48 \pm 6\%$  (n = 3) and 30 mg protease gave  $48 \pm 6\%$  (n = 3). Hence, an increased extraction yield for Se was not observed, and 20 mg protease was used for the extraction of samples. As fish feed is a high lipid matrix, typically constituting 11–35% of oil [33,34], lipase enzymes should potentially increase the release of Se from lipid matrix components. However, the extraction recovery when using lipase in combination with protease was  $43 \pm 14\%$  (n = 3), and compared to the extraction recovery when using protease alone;  $48 \pm 6\%$  (n = 3), there were no increase in the extraction yield. Consequently lipase was not added to the extraction solvent when extracting Se from the diets. These results clearly indicate that most of Se



**Table 2**

Selenium concentrations (mg/kg ww, mean  $\pm$  SD, n = 3) in sample and extracts, extraction yield for Se (%) in the protease extracts and alkaline extracts, the calculated column recovery (%), Selenomethionine (SeMet) and Se(IV) concentration (mg/kg ww, mean SD, n = 3) and the abundance (%) of SeMet and Se(IV) compared to total Se in samples.

Sample type	Sample	Total Se (mg/kg ww)	Total Se in extracts (mg/kg ww)	Extraction yield (%)	Column recovery (%) <sup>a</sup>	SeMet concentration (mg/kg ww)	SeMet abundance (%)	Se(IV) concentration (mg/kg ww)	Se(IV) abundance (%)
Certified reference material	ERM-BB422	1.3 $\pm$ 0.1	1.37 $\pm$ 0.06 <sup>b</sup>	103 $\pm$ 4	75	1.4 $\pm$ 0.1	107		
	Selm-1	2031 $\pm$ 70	1802 $\pm$ 48 <sup>c</sup>	89 $\pm$ 2	117	1271 $\pm$ 64	63		
Diets	Basal low	0.35 $\pm$ 0.02	0.4 $\pm$ 0.1 <sup>c</sup>	114 $\pm$ 29	33	0.09 $\pm$ 0.01	26		
	Inorganic Se low	1.10 $\pm$ 0.03	0.52 $\pm$ 0.02	47 $\pm$ 2	17	0.06 $\pm$ 0.02	5		
	Inorganic Se low – alkaline extract		0.9 $\pm$ 0.2 <sup>c</sup>	82 $\pm$ 18 <sup>c</sup>				0.23 $\pm$ 0.02	21
	Inorganic Se high	15.0 $\pm$ 0.5	6.0 $\pm$ 1.0	37 $\pm$ 8	45	0.12 $\pm$ 0.03	0.8		
	Inorganic Se high – alkaline extract		14 $\pm$ 4 <sup>c</sup>	92 $\pm$ 28 <sup>c</sup>				13.0 $\pm$ 0.03	87
	Organic Se low	2.10 $\pm$ 0.05	1.6 $\pm$ 0.1	76 $\pm$ 5	43	0.59 $\pm$ 0.02	28		
	Organic Se high	15.0 $\pm$ 0.2	13.2 $\pm$ 0.4	88 $\pm$ 3	51	6.1 $\pm$ 0.2	41		
	Basal high	0.89 $\pm$ 0.03	0.57 $\pm$ 0.03	64 $\pm$ 3	49	0.19 $\pm$ 0.02	21		
	Basal low	0.12 $\pm$ 0.01			116	0.11 $\pm$ 0.01	92		
	Inorganic Se low	0.14 $\pm$ 0.01			121	0.12 $\pm$ 0.02	86		
Muscle tissue	Inorganic Se high	0.46 $\pm$ 0.07	0.45 $\pm$ 0.02	90 $\pm$ 4	67	0.12 $\pm$ 0.01	26		
	Organic Se low	0.43 $\pm$ 0.04			118	0.43 $\pm$ 0.02	100		
	Organic Se high	3.4 $\pm$ 0.2	3.6 $\pm$ 0.1	106 $\pm$ 3	132	4.0 $\pm$ 0.9	118		
	Basal high	0.22 $\pm$ 0.03 <sup>c</sup>				0.20 $\pm$ 0.01	91		
	Commercial samples <sup>d</sup>	0.38 $\pm$ 0.02 <sup>c</sup>	0.42 $\pm$ 0.04	110 $\pm$ 11	109		53		

<sup>a</sup> Column recovery(%) is calculated as the sum of quantified peaks expressed as a percentage of the total Se concentration of the extract, where the Se peaks are quantified using the Se standard closest in retention time.

<sup>b</sup> n = 5.

<sup>c</sup> n = 2.

<sup>d</sup> Salmon muscle tissue from the Surveillance program on farmed Atlantic salmon.

in the diet is protein-bound.

The degree of protein degradation in the samples was not measured, but the high extraction yield for Se from the selenised yeast reference material (Selm-1; 89  $\pm$  2% and ERM-422; 103  $\pm$  4%), diets without inorganic Se supplementation (64–114%) and muscle of Atlantic salmon (90–110%) indicate that protease type XIV was efficient in extracting protein-bound Se (Table 2). The variable extraction yields seen for Se in the enzymatic digest of the diets, ranging from 37% to 114% (Table 2), was further evaluated by performing a sequential extraction step with protease for selected diets to increase the Se extraction yield (Supplementary data, S-Table 2). The additional extraction steps only slightly increased the extraction yield for Se; 7–13% (second extraction step) and 1–4% (third extraction step). These results show that the repeated hydrolysis is not effective and not needed. Also, the results indicate that the extraction yield depends on the Se supplementation source, with a lower extraction yield obtained for the inorganic Se diets (37–47%), compared to the organic Se diets (76–88%) when using enzymatic digestion with protease type XIV (Table 2).

Alkaline solvents have previously been used for the extraction of Se in diets supplemented with inorganic Se [18,35]. Therefore, a weak alkaline solution (0.1 M NaOH) was applied for the extraction of Se in the inorganic diets. When using the alkaline solution, the extraction yield for Se increased to 82% and 92% for the inorganic low diet and the inorganic high diet, respectively (Table 2). These results show that NaOH is more suited for the extraction of inorganic Se species compared to enzymatic cleavage. Further, this shows that two separate methods are required; one for the determination of organic Se species using enzymatic extraction and one for the determination of inorganic Se species using a weak alkaline extraction.

### 3.2. Selenium speciation analysis of feeds

Selenium speciation analysis was performed using different chromatographic methods (Supplementary, S-Table 1), where SeMet was determined by cation-exchange HPLC-ICP-MS (Fig. 1a and b) and inorganic Se species were determined by anion-exchange HPLC-ICP-MS (Fig. 1c). In addition, SeCys was analysed for in selected muscle samples by derivatisation and analysis by reversed phase HPLC-ICP-MS (S-Table 1, S-Fig. 1).

No commercial certified reference materials are currently available for Se species in food or feed. For quality control, Selm-1, a certified reference material of selenised yeast, with certified reference values for total Se (2031  $\pm$  70 mg Se/kg dw) and for SeMet (3190  $\pm$  260 mg SeMet/kg dw, i.e. 1268  $\pm$  103 mg Se/kg dw) was used for determination of accuracy and repeatability of the methods. The recovery for SeMet in Selm-1 was in good agreement, 100  $\pm$  5%, (mean  $\pm$  SD, n = 5), with measured values of 1271  $\pm$  64 mg Se/kg dw (mean  $\pm$  SD, n = 5) when using the cation-exchange HPLC-ICP-MS method. The recovery for SeMet in Selm-1 was 89  $\pm$  8% (mean  $\pm$  SD, n = 7) when using the anion-exchange method (measured values of 1131  $\pm$  101 mg/kg dw, mean  $\pm$  SD, n = 7). The cation-exchange HPLC-ICP-MS method was used for the quantitative analysis of SeMet in feeds and in salmon muscle (Table 2). For the determination of Se(IV) and Se(VI) in fish muscle and in feed, the accuracy of the method was verified by spiking experiment to the sample extracts. The recovery of the spiking was 110  $\pm$  17% (n = 7) and 107  $\pm$  6% (n = 7) when spiking with standards for Se(IV) and Se(VI), respectively.

From the speciation analysis, it was seen that SeMet was the major Se species in the enzymatic extracts of the un-supplemented basal low diet (low fish meal inclusion) and basal high diet (high fish meal inclusion), where the SeMet accounted for 26% and 21% of the total Se, respectively (Table 2). However, SeMet accounted for 66% and 71% of

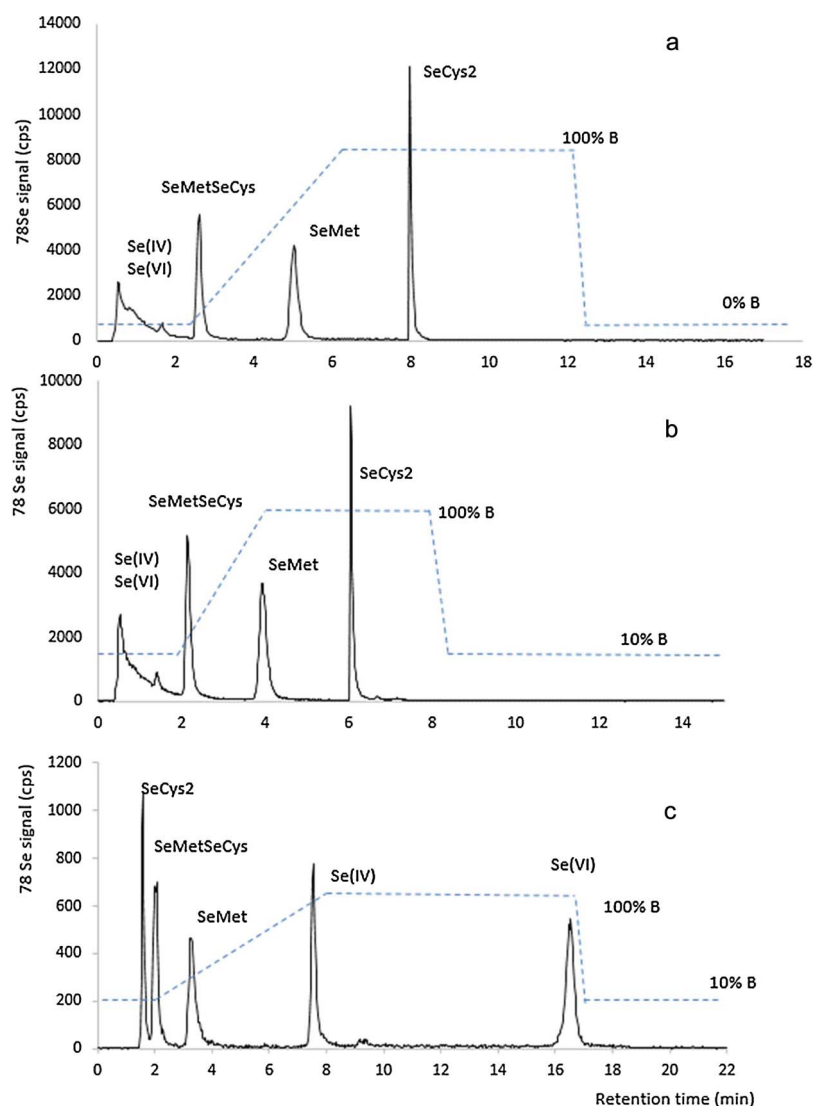


Fig. 1. Chromatograms of a standard mixture (100  $\mu\text{g Se/L}$ ) of selenite (Se(IV)), selenate (Se(VI)), Se-methyl-seleno-cysteine (SeMetSeCys), selenomethionine (SeMet) and selenocystine (SeCys<sub>2</sub>) analysed by cation-exchange HPLC-ICP-MS (a and b) and by anion-exchange HPLC-ICP-MS (c). Different elution gradients were applied for analysis of muscle tissue (a) and diets (b) by cation-exchange HPLC-ICP-MS. The elution gradients are shown for the figures.

the Se peaks detected (by area of chromatographic peaks; data not shown), respectively. The relative low column recovery seen for the diets when analysed by cation-exchange HPLC-ICP-MS (Table 2) is reflecting the low proportion of Se species determined in the diets, and could suggest that the column recovery is affected by the sample type. SeMet in these diets is likely to originate from the marine feed ingredients, and mainly fish meal (accounting for 8 and 15% in the basal diet low and high, respectively). Fish meal generally contains high levels of Se [3], and SeMet is considered the major Se species in marine ingredients [26]. SeMet was the major Se species in the diets supplemented with low and high organic Se (as Se yeast), accounting for 28% and 41% of total Se, respectively (Table 2). Similarly, to the basal diets, SeMet was the major Se species detected in the extracts, accounting for 83% and 89% of the total Se peaks detected, respectively (data not shown). Selenised yeast contains SeMet as the major Se species [36], hence, the presence of SeMet in the organic Se diets was expected. Other minor Se peaks detected in this diet is likely to originate from the Se-yeast [37].

In the inorganic Se diets, low levels of SeMet were detected, with concentrations of  $0.06 \pm 0.02$  and  $0.12 \pm 0.03$  mg/kg feed for the low and high diet (Table 2), respectively. In the enzymatic extract of the inorganic Se high diet, several Se peaks were detected when analysed

both by anion-exchange and cation-exchange HPLC-ICP-MS (Supplementary S-Fig. 2). However, the SeMet peak accounted for only 5% of the total Se peaks detected in the protease extract (data not shown) and interestingly, Se(IV) was not detected in the enzymatic extract. Subsequent spiking experiments on the extract with standards of Se(IV) and Se(VI) gave a recovery in the range of 98–106% (Supplementary data, S-Fig. 3), suggesting that inorganic Se does not complex with matrix components in the final enzymatic extracts. In contrast to the proteolytic extraction, application of a weak alkaline solution (0.1 M NaOH) for the extraction of the inorganic Se diets, Se(IV) was detected as the major Se species in both the inorganic Se low diet and in the inorganic Se high diet when using anion-exchange HPLC-ICP-MS (Fig. 2). A minor peak for Se(VI) was also detected in the alkaline extracts of the samples (Fig. 2). However, Se(VI) was similarly detected in the blank sample (data not shown), suggesting a minor impurity of Se in the extracting solvent, which was compensated for in the quantitative determinations by subtracting the blank signal. Using an external calibration curve for the quantitative determination of inorganic selenium in the extracts, Se(IV) accounted for 21% and 87% of the total Se in inorganic Se low and inorganic Se high diet, respectively (Table 2).

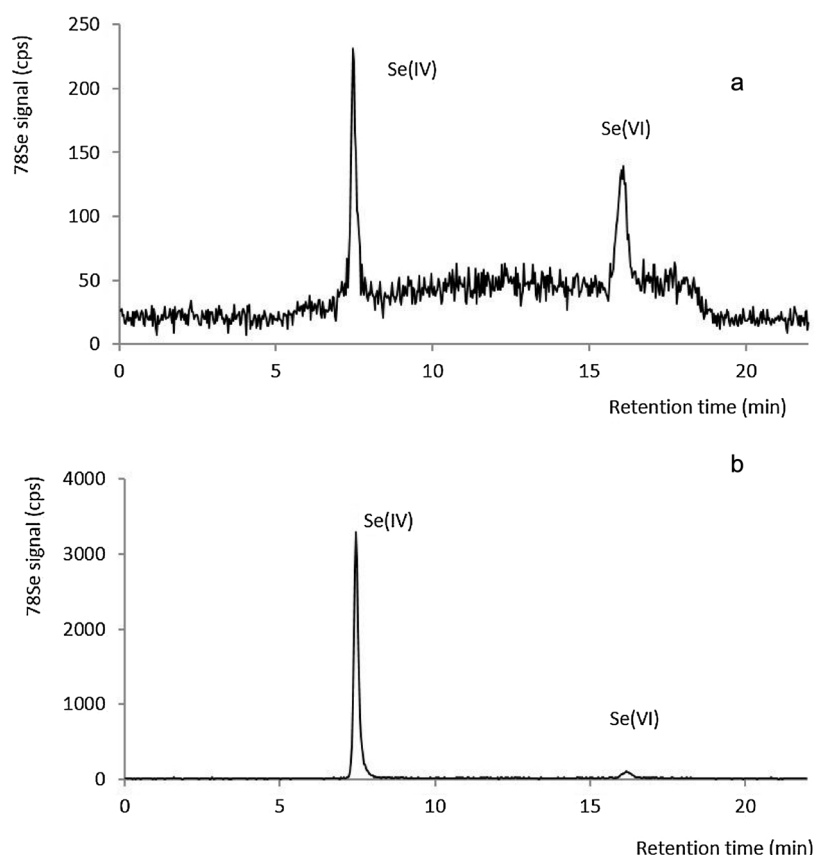


Fig. 2. Chromatograms of the alkaline extracts of inorganic Se low diet (a) and inorganic Se high diet (b) analysed by anion-exchange HPLC-ICP-MS. Please note the different scales on the y-axis.

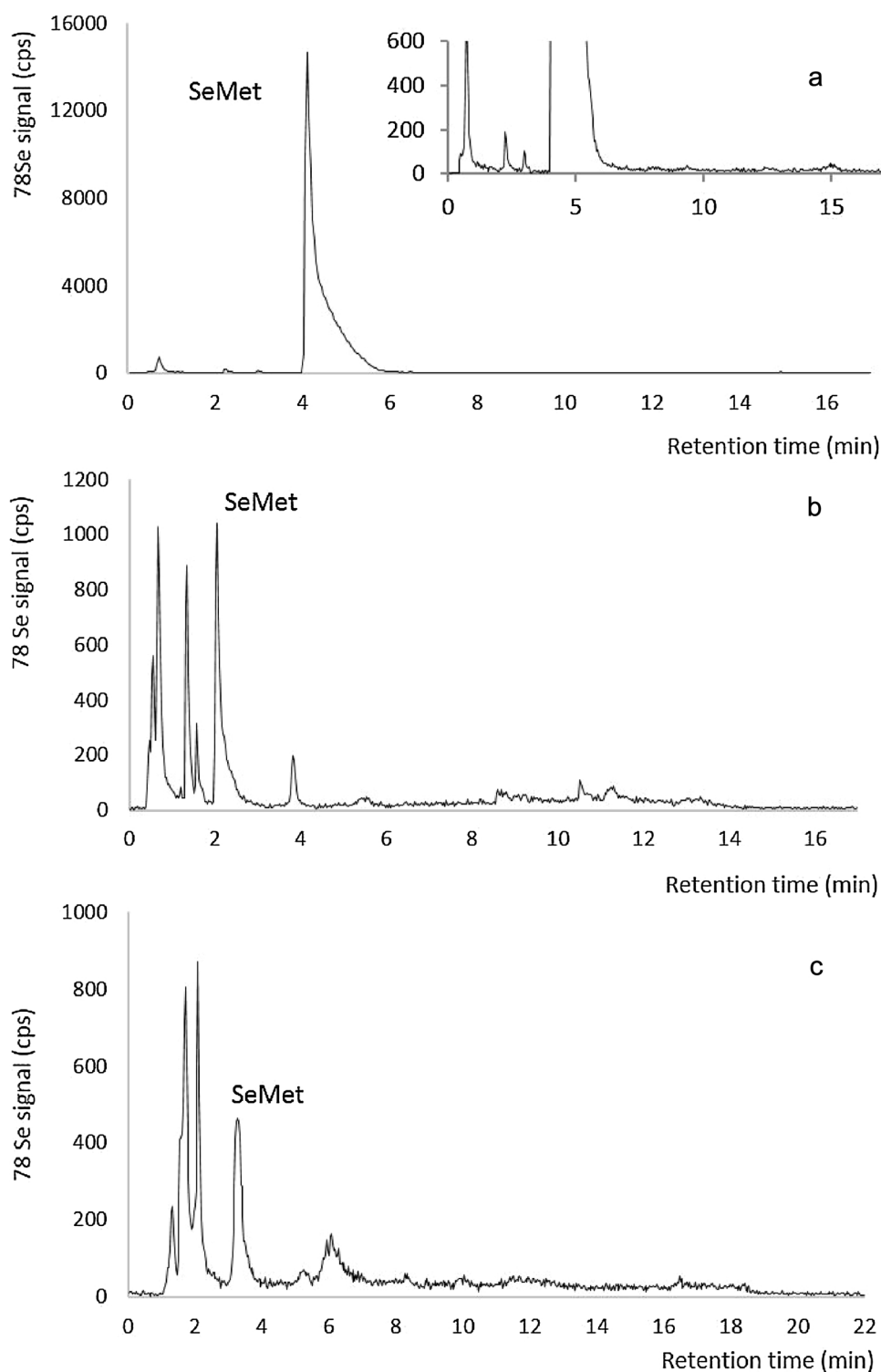
### 3.3. Selenium and Se species in muscle

The accumulation of Se in muscle after 12 weeks of feeding depended on the concentration in feed and on the chemical form of the supplemented Se (Table 1). Higher levels of Se were found in muscle of fish fed diets supplemented with organic Se;  $0.43 \pm 0.04$  (low diet) and  $3.4 \pm 0.2$  (high diet) mg Se/kg ww ( $n = 3$ ), respectively, compared with fish fed diets supplemented with inorganic Se;  $0.14 \pm 0.01$  (low diet) and  $0.46 \pm 0.07$  (high diet) mg Se/kg ww ( $n = 3$ ), respectively. In comparison, muscle of fish fed the basal low and basal high diets contained  $0.12 \pm 0.01$  and  $0.22 \pm 0.03$  mg Se/kg ww ( $n = 3$ ) (Table 1), respectively. A higher retention of Se was seen in muscle of Atlantic salmon fed diets supplemented with organic Se (low:  $21.2 \pm 1.0$  and high:  $24.2 \pm 1.3\%$ ,  $n = 3$ ), than in muscle of salmon fed diets supplemented with inorganic Se ( $13.9 \pm 2.6$  and  $3.7 \pm 0.2\%$ ,  $n = 3$ ) (Table 1). These results suggest that supplemented Se is available to the Atlantic salmon and that organic Se appears to have a higher availability than inorganic Se. The observation is in agreement with earlier findings that SeMet is more readily available than inorganic Se species [6,18,38], and that SeMet supplementation with selenised-yeast or SeMet leads to increased levels of Se in fish, e.g. rainbow trout [18] and Atlantic salmon [39]. In Atlantic salmon fed the basal low and the basal high diets the retention of Se in muscle were  $15 \pm 1$  and  $70 \pm 4\%$  ( $n = 3$ ), respectively. The basal high diet contained more fish meal and krill meal, and less plant protein, than the basal low diet. The higher retention in the salmon fed the basal high diets suggests that Se in marine protein sources is more available than Se in plant protein. This is supported by the work of Godin et al. [18], where higher whole body Se levels were found in rainbow trout fry fed a diet based on marine feed ingredients compared to fry fed a plant based diet, indicating a higher bioavailability of Se in marine ingredients.

Muscle of Atlantic salmon fed the basal diets contained SeMet as the major Se species, accounting for 91 and 92% of the total Se in the samples (Table 2). The SeMet concentration in the extracts were  $0.11 \pm 0.01$  and  $0.20 \pm 0.01$  mg/kg ww (mean  $\pm$  SD,  $n = 3$ ) for the basal low and high, respectively, when using the cation-exchange HPLC-ICP-MS method (Table 2). In comparison, when using the anion-exchange method, the SeMet concentrations were  $0.06 \pm 0.2$  and  $0.13 \pm 0.05$  mg/kg ww (mean  $\pm$  SD,  $n = 3$ ) for the basal low and high, respectively (data not shown). The lower SeMet concentration detected by the anion-exchange method compared to the cation-exchange HPLC-ICP-MS method for the muscle samples is consistent with the results for the certified reference materials Selm-1 and ERM-BB442. The somewhat lower results for SeMet obtained with the anion-exchange method may be explained by matrix effects that could cause a reduction of the Se signal in the ICP-MS.

SeMet was also the major Se species in muscle of salmon fed diets supplemented with organic Se, with concentrations of  $0.43 \pm 0.02$  and  $4.0 \pm 0.9$  mg/kg ww accounting for 80% and 99% of the Se peaks detected in the fish fed organic low and high diets, respectively (Table 2, Fig. 3a and S-Fig. 4a for organic Se high). Other minor Se species, eluted in the void and in the retention time area of SeMetSeCys (Fig. 3a), suggesting the presence of SeMetSeCys and other Se species with similar ionic properties. SeMet is considered the major Se species in muscle of marine fish, including salmon [1,22]. In contrast, the muscle of Atlantic salmon fed high inorganic Se diet, SeMet accounted for only 26% of the total Se in the sample (Table 2, Fig. 3b and S-Fig. 4b). The salmon fed inorganic Se diets did not contain elevated levels of SeMet compared to salmon fed the basal diets (Table 2), which is consistent with the suggested metabolic pathway for Se in animals, where SeMet cannot be synthesized from selenite [40]. However, several unidentified peaks were detected in the muscle of Atlantic salmon fed the inorganic Se high diet both when analysed by cation-exchange





**Fig. 3.** Chromatogram of protease extract of muscle of Atlantic salmon fed organic Se high diet, analysed by cation-exchange HPLC-ICP-MS (a), the enlargement shows the presence of minor Se peaks in the sample extract, and of muscle of Atlantic salmon fed inorganic Se high diet, analysed by cation-exchange HPLC-ICP-MS (b) and by anion-exchange HPLC-ICP-MS (c).

and anion-exchange HPLC-ICP-MS (Fig. 3b and c). Selenite and Se(VI) were not detected in the muscle of the salmon fed the inorganic Se high diet when analysing the protease (Fig. 3c) and alkaline (data not shown) extracts by anion-exchange HPLC-ICP-MS. These results are consistent with the findings of Godin et al. [18] where SeMet were detected in whole body rainbow trout fry fed inorganic Se supplemented feed. Furthermore, unidentified Se species were similarly detected in rainbow trout fry fed inorganic Se supplemented feed [18]. However, the trout contained a nearly uniform proportion of unknown

Se species (24–38%), irrespectively of being fed inorganic or organic Se supplemented diets [18].

Selenite is metabolised to hydrogen selenide, and further to SeCys, which is specifically incorporated into selenoproteins, mainly in liver [40]. The high dietary level of selenite could lead to the presence of SeCys in the liver, and possibly in the muscle tissue. Analysis of SeCys is challenging due to the highly reactive free selenol group, which cause the species to rarely exist in its free form [41], and requires stabilisation through derivatization prior to analysis [16]. Samples were derivatised

and results indicate that SeCys is present in muscle of fish fed high inorganic diet, however, it is a minor Se species (Supplementary, S-Fig. 1). In wild-caught fish, e.g. sand whiting (*Sillago ciliata*), leather jacket (*Monacanthus chinensis*) and luderick (*Girella tricuspidata*) low levels of SeCys (from 4 to 17% of total Se) have been found [22]. Similarly, somewhat higher levels of SeCys (from 20 to 30% of total Se) were observed in trout fry, irrespective of whether they were fed diets supplemented with inorganic Se or organic selenised yeast [18]. Other Se species could account for the unidentified Se compounds in muscle of the salmon fed inorganic high diet, such as selenoneine. Selenoneine (2-selenyl-N $\alpha$ -N $\alpha$ -N $\alpha$ -trimethyl-L-histidine) was first identified in blood and muscle of bluefin tuna (*Thunnus orientalis*) [42], and has later been found in muscle of a range of fish [43,44], often as a minor peak. Yamashita et al. [42] hypothesised that selenite can be metabolised to selenoneine. Hence, selenite, in excess, could lead to formation of selenoneine, which is accumulated in muscle. Further investigations are needed to determine the possible metabolism of selenite to selenoneine in Atlantic salmon.

#### 4. Conclusion

Se species were analysed using anion-exchange HPLC-ICP-MS for inorganic Se species and cation-exchange HPLC-ICP-MS for organic Se species. SeMet, Se(IV) and Se(VI) were analysed in fish feeds and Atlantic salmon using these methods. Extraction of Se species from fish feed was optimised, and two methods were applied, enzymatic cleavage for organic Se supplemented feeds and weak alkaline solvent for inorganic Se supplemented feeds. In addition, selected muscle samples were analysed for SeCys using extraction procedure including derivatisation and analysis by reversed-phase HPLC-ICP-MS. Supplementation of fish feed with organic and inorganic Se sources have different effects on the retention of Se and the Se species retained in the muscle tissue of Atlantic salmon. Higher retention of Se was seen in Atlantic salmon fed diets supplemented with organic Se than with inorganic Se. Speciation analysis confirmed that SeMet was the major Se species in muscle of Atlantic salmon fed both basal diets and diets supplemented with selenised yeast. Several unidentified Se peaks were detected in muscle of salmon fed high inorganic Se diet, and analysis showed that SeCys is a minor Se species present in this fish muscle tissue.

#### Conflict of interest

The authors declare no conflict of interest.

#### Acknowledgements

We wish to thank Siri Bargård (NIFES) for organizing the sampling of fish, Mali Bjerkhaug Hartviksen and Terje Utne (EWOS Innovation) for conducting the feeding trial, and Marie Hillestad (BioMar AS) for producing the diets. This study was financed by the Norwegian Seafood Research Fund (FHF), EWOS AS/Cargill Aqua Nutrition, Biomar AS, Skretting AS and Marine Harvest ASA (FHF project no. 900871).

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jtemb.2018.02.005>.

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